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Photocontrol of Cell Adhesion Processes: Model Studies with Cyclic Azobenzene-RGD Peptides

Brief Communication

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Summary

A photoresponsive integrin ligand was synthesized by backbone-cyclization of a heptapeptide containing the integrin binding motif Arg-Gly-Asp (RGD) with 4-(aminomethyl)phenylazobenzoic acid (AMPB). Surface plasmon enhanced fluorescence spectroscopy showed that binding of the azobenzene peptide to $\alpha_v\beta_3$ integrin depends on the photoisomeric state of the peptide chromophore. The higher affinity of the *trans* isomer could be rationalized by comparing the NMR conformations of the *cis* and *trans* isomers with the recently solved X-ray structure of a cyclic RGD-pentapeptide bound to integrin.

Introduction

Cell adhesion is a crucial process in the assembly of individual cells into the three-dimensional architecture of animal tissues [1]. Although stable cell interactions are required for the structural integrity of tissues, active adhesion mechanisms are essential for the proper functionality of many physiological processes such as embryogenesis, cell differentiation, haemostasis, wound healing, and immune responses, but also for pathophysiological events such as tumor cell extravasation or invasion [2–4]. These cell adhesion processes are mediated by cell-surface receptors, among which integrins represent the most diverse and prominent family [5–7]. As a main integrin-ligand motif, the tripeptide sequence Arg-Gly-Asp (RGD) has been identified; it is found in most of the matrix proteins [1, 8, 9], and selectivity of these molecular recognition processes is dictated by additional ligand epitopes. Moreover, conformational features, i.e., differentiated spatial displays, of the RGD motif are decisive for both affinity and selectivity assessed with conformationally restricted cyclic RGD peptides [10–12]. This marked conformation-dependent binding affinity of RGD ligands for integrins lends itself to a photocontrol of this molecular recognition process by the use of suitable photoresponsive RGD constructs.

Azobenzene has been extensively used in the past as a versatile light switch for reversible photomodulation of a wide spectrum of physical and biophysical properties [13,14] including conformational states of model peptides [15–17]. In previous studies, we have successfully exploited a backbone-incorporated azobenzene moiety for reversible *cis/trans* photoisomerization of the conformational states of constrained cyclic peptides [18–21]. In the present study, this concept was applied for the design of integrin ligands, and with the cyclic azobenzene peptide *c*[-Lys[1]-Ala[2]-Arg[3]-Gly[4]-Asp[5]-D-Phe[6]-Val[7]-AMPB-] (**1**), photomodulation of binding affinity for $\alpha_v\beta_3$ integrin was successful.

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Results and Discussion

Based on our previous experiences with (4-amino)phenylazobenzoic acid and (4-aminomethyl)phenylazobenzoic acid (AMPB) as cyclic peptide-backbone constituents [22], modeling experiments were suggesting that cyclization of hexapeptides containing the RGD motif in permutated sequence positions should be feasible, at least when using the more flexible AMPB derivative. The sequence of the hexapeptide was adapted to the well-known $\alpha_v\beta_3$ integrin ligand *c*[-Arg-Gly-Asp-D-Phe-Val-] [23, 24] by adding a lysine residue for possible side-chain labeling. However, all synthetic efforts failed even when applying different cyclization sites and macrolactamization procedures. By expanding the ring size with an additional amino acid residue, cyclization of the side-chain-protected H-Asp(OtBu)-D-Phe-Val-AMPB-Lys(Boc)-Ala-Arg(Boc)$_2$-Gly-OH with PyBOP/HOBt was successful, and upon deprotection with TFA/H_2O (95:5) the target peptide **1** was obtained as an analytically well-characterized compound.

Cis/trans photoisomerization of the cyclic peptide **1** in aqueous solution by irradiation at 360 and 450 nm, respectively, is a fully reversible process (*trans* isomer, λ_{max} = 335.5 nm; *cis* isomer, λ_{max} = 253.4 nm). After relaxation at 50°C in the dark, ^{1}H-NMR spectra were consistent with the presence of almost exclusively *trans*-azobenzene isomer (>99%), whereas upon irradiation at 360 nm a maximum content of ~80% of *cis* isomer could be obtained in the photostationary state, as derived from the NMR spectra. This fully agrees with previous results from cyclic azobenzene peptides [18–21]. The NMR spectra of the two isomers are characterized by distinct sets of resonances. These differ in the chemical shifts to extents that allow unambiguous assignment even for the *cis* isomer in the photostationary state. From the NOESY spectra, sufficient interproton distance constraints could be extracted to define the structural preferences of the two isomers in aqueous solution. The smaller ring size of the RGD peptide compared to the cyclic azobenzene peptides of our previous studies results in a highly frustrated system for the *trans*-azobenzene isomer which flip-flops between conformational substates with two different orientations of the azobenzene moiety and the peptide portion Lys[1]-Ala[2]-Arg[3]-Gly[4]. These substates are linked by independent hinge motions (around Val[7]/Lys[1] and Lys[1]/Gly[4]) (Figure 1). Photoisomerization to the *cis*-isomer relaxes the system into a larger conformational space occupied by an ensemble

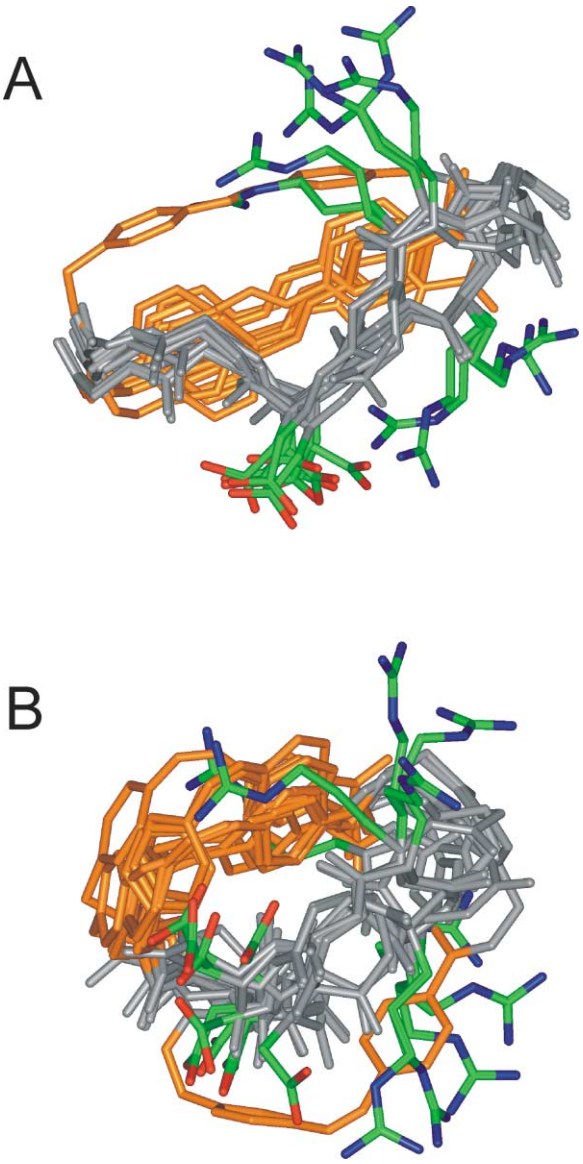

Figure 1. NMR Structural Ensembles for Both Isomers of the Azobenzene Peptide **1**

Stick models of the ten lowest energy structures of *c*[-Asp-D-Phe-Val-AMPB-Lys-Ala-Arg-Gly-] in the *trans* (A) and *cis* conformation (B). The peptide backbone is colored gray, the AMPB moiety is orange, and the Arg and Asp side chains are in atom colors.

of structures that differ significantly in the peptide-backbone fold and, correspondingly, even in the spatial display of the RGD side chains.

We have recently developed a peptide-tethered artificial lipid membrane system with functionally incorporated integrins that allows monitoring of integrin-ligand interactions by surface plasmon enhanced fluorescence spectroscopy (SPFS) [25]. This system proved well suited for analyzing differentiated binding affinities of synthetic heterotrimeric collagen peptides containing the $\alpha_1\beta_1$ integrin binding epitope of collagen type IV [26]. For the purposes of the present study, $\alpha_v\beta_3$ integrin was incorporated into the artificial lipid membrane, and the azobenzene peptide **1** was fluorescence labeled with Cy5 at the side-chain amino group of the Lys residue. The binding affinities of the cyclic peptide **1** in the *trans* and *cis* conformation were assayed with this experimental set-up. As shown in Figure 2, the *cis* isomer was found to exhibit a significantly lower binding affinity than its *trans* isomer ($-40\% \pm 10\%$). Selective binding of the ligand to the integrin was assessed by its reversal with EDTA, since Mn^{2+} is an essential proadhesive cation. A similarly Cy5-labeled derivative of the well-established $\alpha_v\beta_3$ integrin ligand *c*[-Asp-D-Phe-Val-Lys-Arg-Gly-] [32] was used as positive control. In this system, it exhibits a binding affinity of the same order of magnitude, whereas no fluorescence was detected with the Cy5-labeled cyclic azobenzene peptide *c*[Lys-AMPB-Lys-Cys-Ala-Thr-Cys-Asp-Lys-Lys] that lacks the RGD motif as negative control. In situ photoisomerization of the integrin ligand peptide is hampered by adverse interferences of the Cy5 dye.

Initial modeling studies based on the known structural preferences of $\alpha_v\beta_3$ integrin ligands [11] predicted an easier fit of the *cis* conformer into the integrin binding pocket. However, the experimental findings clearly revealed higher binding affinity of the *trans* isomer with its peptide chain in a rather stretched parallel alignment to the azobenzene moiety. To rationalize this observation, the coordinates of the crystal structure of the extracellular portion of $\alpha_v\beta_3$ integrin complexed with the cyclic pentapeptide *c*[-Arg-Gly-Asp-D-Phe-(Me)Val-] (Protein Data Bank entry 1L5G) [27] were used to match the ligand conformations (Figure 3). Although not in optimal mode, the RGD portion of the cyclic *trans*-azobenzene peptide displays the essential Asp and Arg side chains in a similar mode for interaction with the protein binding pocket. However, the chain reversal at the D-Phe-Val sequence portion as imposed by the *trans* azobenzene conformation would clash with a protein loop unless some flexibility in the peptide or the protein counterpart allows for better adaptation of this ligand. On the other hand, the entropic cost for binding of the *cis* isomer with its larger conformational space is unfavorable and may thus account for the weaker binding already reported for conformationally nonrestricted RGD peptides [10–12].

Significance

The experimental results have validated the concept of conformationally restricted azobenzene peptides containing the ubiquitous RGD integrin binding motif for photomodulation of adhesion processes. With the structural information now available from the crystallographic analysis of the $\alpha_v\beta_3$ integrin/cyclic RGD-peptide complex and the NMR conformational analysis of the cyclic azobenzene peptide, amelioration of binding affinities can be envisaged by modification of the sequence composition as well as extension of the ring size. Similarly, changes in the experimental set-up of the surface plasmon spectroscopy may allow bypass of the use of interfering fluorescent dyes and thus in situ photomodulation of the integrin binding affinities, with the ultimate goal of a photocontrol of cell adhe-

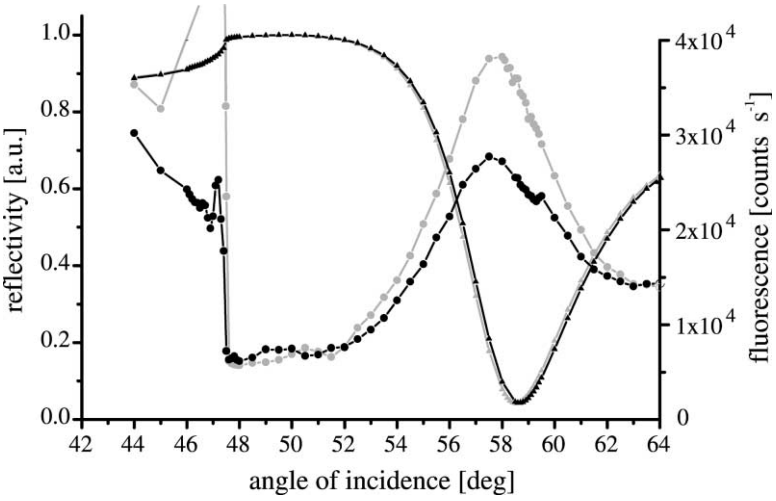

Figure 2. Integrin Binding of Azobenzene Peptide **1**

A representative experiment is shown for the SPFS binding assay of fluorescence-labeled peptide **1** using an artificial $\alpha_v\beta_3$ integrin membrane preparation coated on a gold surface. Background signals are subtracted from the fluorescence scans. The fluorescence scans corresponding to the integrin with bound peptide **1** as *cis* (black circles) and *trans* isomer (gray circles) are shown; likewise, the plasmon scans upon binding of *cis* (black triangles) and *trans* isomer (gray triangles) are reported. In the fluorescence scan of the bound *trans* isomer, an artifact due to internal reflections is present between 46° and 47° angles of incidence.

sion for time-resolved monitoring of associated cellular processes.

Experimental Procedures

Synthesis

Details of the synthesis of the cyclic RGD peptide with AMPB as backbone constituent and its analytical and spectroscopic characterization will be reported elsewhere.

NMR Conformational Analysis

NMR spectra of the azobenzene peptide were recorded in water and at 303 K on a Bruker DRX 500 spectrometer equipped with pulsed-field-gradient (PFG) accessories at a proton frequency of 500.13 MHz. Resonance assignments were performed according to the method of Wüthrich [28]. The 2D TOCSY was recorded with a spin-lock period of 75 ms using the MLEV-17 sequence for isotropic mixing [29]. Experimental distance constraints (*trans* isomer, 44; *cis* isomer, 56) were extracted from 2D ROESY [30] experiments with a mixing time of 100 ms. Angle constraints (*trans* isomer, 5; *cis* isomer, 6) were extracted from 2D DQF-COSY [31] and simple 1H-1D spectra. Structure calculations and evaluations were performed with the INSIGHTII 2000 software package (Accelrys, San Diego) as described previously [19]. No significant violations of experimental constraints occurred for any of the calculated structures.

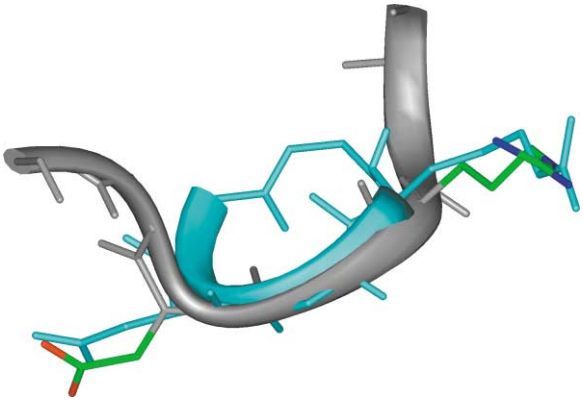

Figure 3. Side Chain Orientation of Complexed *c*[RGDf(Me)V] and Free *c*[RGDfV-AMPB-KA]

Superposition of one preferred conformation of the *trans* isomer (gray) of *c*[-Asp-D-Phe-Val-AMPB-Lys-Ala-Arg-Gly-] with the X-ray structure (light blue) of *c*[-Arg-Gly-Asp-D-Phe-MeVal-] when complexed to $\alpha_v\beta_3$ integrin [27]. The backbone conformation of both peptides is highlighted by gray and light blue ribbons, respectively. The side chains of Arg and Asp are colored light blue for the pentapeptide and in atom colors for the azobenzene peptide.

SPFS Measurements

SPFS binding experiments were carried out using a self-assembled set-up with $\alpha_v\beta_3$ integrin (Chemicon Inc., Temecula, Ca) embedded into a DMPE/PC bilayer coated to the gold surface via a hydrophilic laminin-peptide layer as described elsewhere [25]. The cyclic azobenzene-peptide **1** and *c*[-Asp-D-Phe-Val-Lys-Arg-Gly-] [32] as well as the oxidized *c*[Lys-AMPB-Lys-Cys-Ala-Thr-Cys-Asp-Lys-Lys] (unpublished synthesis) were fluorescence labeled with Cy5 N-hydroxysuccinimide ester (Amersham Pharmacia, Uppsala, Sweden) via acylation of the Lys side-chain amino group, and quantitative derivatization was assessed by hplc. The fluorescence experiments were performed after incubation of the peptides at room temperature in 50 mM Tris•HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$•$6H_2O$, and 10 mM $MnCl_2$•$2H_2O$ for 30 min followed by rinsing with the buffer. Complex dissociation was achieved by incubation with 0.5 M EDTA. The reported difference in binding affinity between *cis* and *trans* isomer is the average of four independent measurements. Repetitive measurements on the same membrane preparation are possible, but due to aging/deterioration effects we preferred fresh membrane preparation for each experiment.

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